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POTENTIATION OF TNF-MEDIATED CELL KILLING BY MITOXANTRONE

RELATIONSHIP TO DNA SINGLE-STRAND BREAK FORMATION

MONICA VALENTI, GUIDO CIMOLI, GABRIELLA LUCIA MARIANI, PIER FRANCO CONTE,* SILVIO PARODI and PATRIZIA RUSSO†

Istituto Nazionale per la Ricerca sul Cancro, Genova, and *Ospedale Santa Chiara, Pisa, Italy

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Abstract—Tumor necrosis factor (TNF) is a pleiotropic cytokine that mediates different cellular responses including cytotoxicity, cytostasis, proliferation, differentiation and expression of specific genes. Recent studies have demonstrated that chemotherapeutic drugs that inhibit the nuclear enzyme DNA topoisomerase II synergize with TNF in tumor cell killing *in vitro* and *in vivo*. We now report that a combination of TNF and the topoisomerase II inhibitor Mitoxantrone produced dose-dependent synergistic cytotoxicity against the human ovarian cancer cell line A2774 in a clonogenic assay (1 hr treatment). This result was obtained with simultaneous administration of the drug and the cytokine under test, and is independent of modification of Mitoxantrone uptake. This combination is responsible for an evident augmentation of "cleavable complex" formation. From isolated nuclei, we have isolated also the topoisomerase II activity; we observed an increment when the cells were previously treated with TNF, 2.5 min before nuclear extraction. After 10–30 min of treatment with TNF, the topoisomerase II activity returned to normal values. If TNF is not given with but 30 min before Mitoxantrone, no potentiation of cytotoxicity or break induction is observed. These results suggest that specific timing of the association may be needed also when attempting to translate it to animals and humans.

The combination of cytokines and cytotoxic drugs offers a new approach to increasing the therapeutic index in the treatment of neoplastic diseases. Unfortunately, there is no consensus on optimal strategies for combining these agents. A better understanding of the mechanisms of action of cytokines and chemotherapeutic drugs should be reached to design clinical trials based on preclinical "rationales". The broad spectrum of chemotherapeutic drugs, whose activity can be enhanced by cytokines [i.e. interferon + 5-fluorouracil [1]; interferon + dacarbazine [2]; interleukin 2 + cyclophosphamide [3]; tumor necrosis factor (TNF‡) + Mitoxantrone [4, 5]; TNF + VP16 [6]; granulocyte colony-stimulating factor + VP16 [7]; granulocyte-macrophage colony-stimulating factor + Mitoxantrone [8]], argues for multiple levels of drug interaction *in vitro*: alteration in the cellular drug uptake, direct modulation of drug-targeted enzymes, changes in metabolism or disposition of a drug, modulation of receptors, modulation of cell cycle phases and modulation of gene expression.

Topoisomerases I and II have emerged as a critical intracellular target of some cytotoxic drugs such as Camptothecin, Doxorubicin, Mitoxantrone and VP16. In cells, these inhibitors produce strand breaks which can be assayed by alkaline elution, performed under deproteinizing conditions [9]. There is convincing evidence that the synergy between TNF and these drugs is related to the ability of TNF to increase topoisomerase-associated strand breaks as well as to increase the level of extractable topoisomerase catalytic activity [10].

In combining cytokines and cytotoxic drugs, the sequence appears crucial for synergistic antiproliferative interactions. The data may be relevant to the planning of future animal and human trials using TNF and topoisomerase-targeted drugs for the treatment of cancer. This report concentrates on the evaluation of the synergistic cytotoxic interaction between TNF and Mitoxantrone.

The *in vitro* potentiation of topoisomerase II-targeted drugs by TNF has been previously documented [4–6, 10]. Recently, clinical trials have been started to determine whether positive therapeutic benefits will result from the association Doxorubicin + TNF [11] or Mitoxantrone + TNF [12] or VP16 + TNF [13]. However, it is clear from *in vitro* studies that variables such as dose, treatment schedule and timing of the combination of TNF and drug subtly modulate the activity of this combination therapy. Manipulation of these parameters may enhance the therapeutic benefits of this chemotherapeutic regimen even further. In this report we have investigated in particular the timing of TNF administration in respect to Mitoxantrone administration.

† Corresponding author: Patrizia Russo, Dept of Chemical Carcinogenesis, Istituto Nazionale per la Ricerca sul Cancro, V. le Benedetto XV, 10 I-16132 Genova, Italy. Tel. (39) 10 3534212; FAX (39) 10 352999.

‡ Abbreviations: TNF, tumor necrosis factor; ICFA, inhibition of colony formation assay; HI-FCS, heat-inactivated fetal calf serum; KDNA, kinetoplast DNA; SSBs, single strand breaks; SDS, sodium dodecyl sulfate; PVC, poly vinyl chloride.

MATERIALS AND METHODS

Cell line. A2774, a human ovarian adenocarcinoma cell line, obtained from a patient suffering from a FIGO stage III ovarian cancer, was utilized for this study (kind gift from Dr S. Ferrini, Dept of Pharmacology, our Institute).

A2774 was maintained as monolayer cultures in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (HI-FCS) and 1% gentamicin. The doubling time is 24 ± 1 hr and the modal chromosome number is 46.

When 2.5×10^7 cells were heterotransplanted i.p. into nude mice abdominal tumors appeared within 10 to 20 days, with formation of abundant ascites before the death of the animal. The histological diagnosis is a serous ovarian epithelioma.

Drugs. Mitoxantrone was purchased from Lederle Italia (Catania, Italy); [^{3}H]Mitoxantrone (3 mCi/mL) was synthesized by the New England Nuclear Corp. (Boston, MA, U.S.A.); [^{3}H]thymidine and [^{14}C]thymidine were purchased from the New England Nuclear Corp.

Human recombinant TNF (rHuTNF) was obtained from Knoll-BASF (Ludwigshafen, Germany). A stock solution of rHuTNF, containing 0.1 mg/mL of protein, was stored at −80°. Specific activity was 8.74×10^6 U/mg protein (48 hr L929 bioassay without actinomycin D, as determined in the Knoll-BASF Laboratory).

Drugs were diluted in RPMI-1640, 10% HI-FCS and used immediately.

Cytotoxicity assay. Drug-induced cytotoxicity was determined by inhibition of colony formation assay (ICFA) (drug treatment for 1 hr). Cells (500/2.5 mL) were plated in six-well Nunc dishes and allowed to attach for 18 hr at 37°. The cells were treated with drug for 1 hr, then the drug was removed by washing (twice) and the cells were allowed to grow for 12–14 days. Colonies were stained with 0.5% methylene blue in 50% methanol and counted. The plating efficiency of the untreated cells was 32.6% and used to normalize for drug-induced cytotoxicity. The IC_{50} value represents the 50% inhibitory concentration and was calculated by linear interpolation of the values immediately higher and lower than 50% inhibition.

Transport studies. The procedure previously described by Pommier *et al.* [14], with minor modifications, was utilized. Exponentially growing A2774 cells were concentrated to approximately 10^7 cells/mL in RPMI 1640 plus 10% HI-FCS. After 30 min incubation with different concentrations of Mitoxantrone containing 0.2 μCi/mL [^{3}H]Mitoxantrone in the presence or absence of TNF (100 U/mL), cells were washed and reincubated in drug-free medium, then 1 mL of the cell suspension was layered above 0.6 mL of silicone oil (Versilube F50, General Electric Co., NY, U.S.A.) in a microcentrifuge tube and centrifuged for 1 min at 12,000 *g*.

Determination of DNA single-strand breaks (SSBs). For the determination of drug-induced DNA breaks in A2774 cells, the alkaline elution technique was used as described by Russo *et al.* [15]. The elution was performed under deproteinizing conditions. Briefly, A2774 cells were seeded (2.5×10^6 cells/150 cm^2) and labeled with [^{3}H]-thymidine (0.2 μCi/mL) for 48 hr. The medium was then changed, and the cells were grown in fresh medium for an additional 4 hr to allow incorporation of the label into high molecular mass DNA. The cells were then exposed to different concentrations of drug, in the presence or absence of TNF, for 1 hr. The drug was removed by washing the cells twice with excess Hanks' balanced salt solution at 4°. Cells were scraped in Hanks' balanced salt solution and DNA SSBs were then assayed by alkaline elution under deproteinizing conditions [15]. DNA SSB frequencies were expressed both in rad equivalents and in terms of $[-\log_r]$, where r is the fraction of DNA retained on filter after elution of 15 mL of eluting solution (fifth fraction). $[-\log_r]$ is directly proportional to the absolute number of SSBs [16]. With this technique both protein-linked and protein-independent breaks were detectable.

Quantification of DNA fragmentation by a filter binding assay. A filter binding assay was performed under non-deproteinizing conditions using protein-adsorbing filters (vinyl/acrylic copolymer filters, Metricel membrane, 0.8 μm pore size, 25 mm diameter; Gelman Sciences) according to Bertrand *et al.* [17]. Cells (0.5×10^6) prelabeled with [^{14}C]-thymidine loaded into PVC filters were lysed with 5 mL of solution containing 0.2% sodium sarkosyl–2 M NaCl–0.04 M EDTA (pH 10.0). After the lysing solution had dripped through by gravity, it was washed from the filter with 10 mL of 0.02 M EDTA (pH 10.0). Filters were then processed as in the case of alkaline elution [15]. Radioactivity was counted by liquid scintillation spectrometry in each fraction (loading, wash, lysing solution + EDTA wash, filter). DNA fragmentation was determined as the percentage of ^{14}C-labeled DNA in the lysing solution fraction divided by the total intracellular ^{14}C-labeled DNA. With this technique only protein-independent breaks were detectable.

Preparation of nuclear extracts from A2774 cells. A2774 cells (0.7×10^7 cells) were seeded into 80 cm^2 flasks and monolayers were established by overnight culture at 37°. Cells were treated with 100 U/mL TNF for various incubation times and harvested by scraping. Nuclear extracts were prepared as described by Drake *et al.* [18]. All extractions were performed at 4°. Nuclear topoisomerase II was extracted by 0.35 M salt on ice for 30 min and sedimented (100,000 *g* for 60 min) and the clear supernatant was removed. To minimize proteolysis during extraction of the nuclear topoisomerase II, the protease inhibitors benzamidine, soybean trypsin inhibitor, leupeptin, pepstatin and aprotinin were included in all buffers. Details are given in Drake *et al.* [18].

Determination of topoisomerase II activity. Topoisomerase II catalytic activity was determined by testing the ability of extracts to decatenate ^{3}H-labeled kinetoplast DNA (KDNA) into free minicircles and assayed quantitatively by centrifugal analysis in which [^{3}H]KDNA was used as substrate and free minicircles were separated from KDNA by a brief centrifugation according to Deffie *et al.* [19]. The radioactivity in the "supernatants" (which contained decatenated KDNA) and the "pellet"

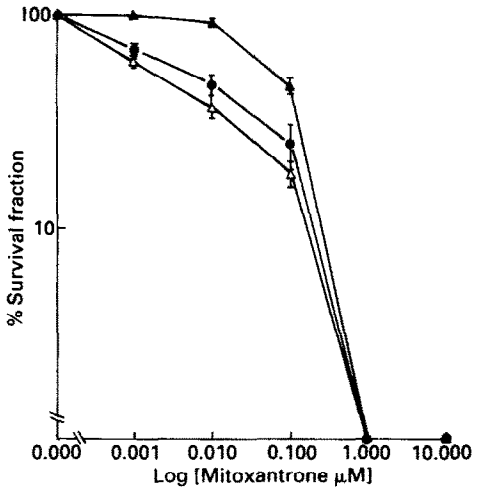


Fig. 1. Relative survival of human ovarian cancer cell line A2774 following exposure to various concentrations of Mitoxantrone alone (▲) or plus TNF 1000 U/mL (●) or 100 U/mL (△). Cells were exposed to Mitoxantrone or Mitoxantrone + TNF for 1 hr at 37° in the clonogenic assay. Data are representative of four or six separate experiments. Bars, ± SE.

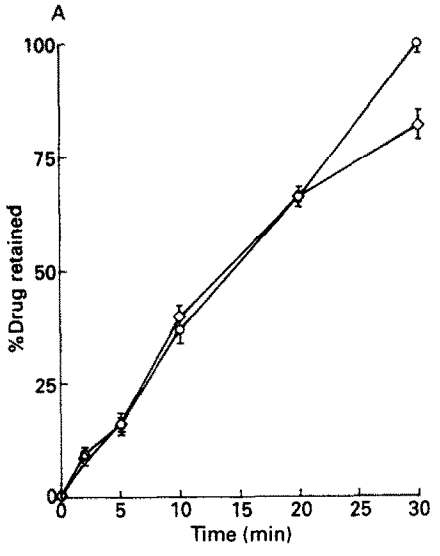
(which contained the remaining KDNA starting material) was then quantified [19].

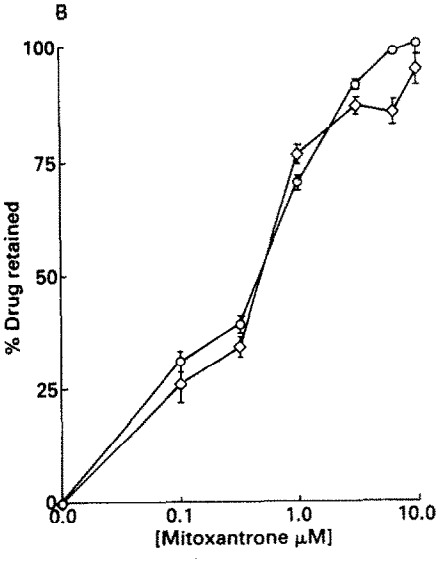
RESULTS

Synergistic cell killing induced by TNF and Mitoxantrone

We have demonstrated previously [4, 5] that TNF enhances the cytotoxicity of Mitoxantrone, in seven ovarian cancer cell lines, when incubated simultaneously with the drug for 20 hr in a crystal violet assay which does not differentiate between cytotoxic and cytostatic effects of drugs. Here we extended our observations by demonstrating potentiation between TNF and the topoisomerase II inhibitor Mitoxantrone in A2774 cells in a clonogenic assay (ICFA).

One hour treatment with TNF alone, at different concentrations (from 10 to 1000 U/mL), did not produce significant cytotoxicity in the A2774 cell line (data not reported). Since, at the highest concentration, TNF (1000 U/mL) exhibited less than





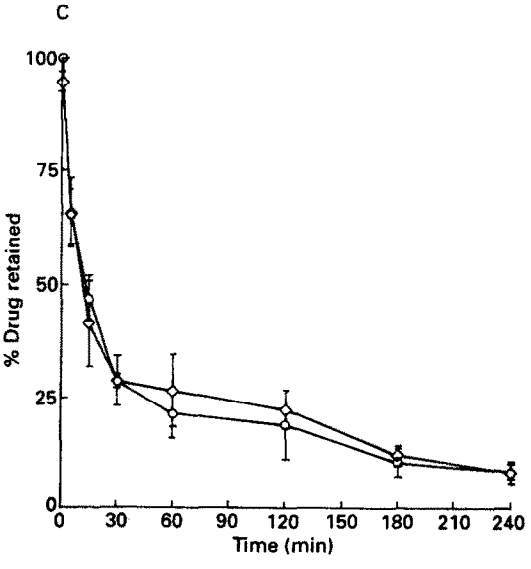


Fig. 2. (A) Dependence of cellular uptake of 10 μM [^{3}H]-Mitoxantrone on time in the absence (○) or presence of TNF 100 U/mL (◇). Representative time course of [^{3}H]-Mitoxantrone (10 μM) accumulation in the absence (○) or presence (◇) of TNF (100 U/mL) in A2774 cells. Data are representative of four or six separate experiments. Bars, ± SE. (B) Dependence of cellular uptake of [^{3}H]-Mitoxantrone on drug concentration in the absence (○) or presence of TNF 100 U/mL (◇). Cells were incubated for 30 min at 37°. Data are representative of four or six separate experiments. Bars, ± SE. (C) Effect of TNF (100 U/mL) on [^{3}H]Mitoxantrone (10 μM) retention in A2774 cells. (◇). With TNF, (○) without TNF. Cells were treated for 30 min at 37°, then drug was removed by washing and cells were incubated in drug-free medium for additional time. Data are representative of four or six separate experiments. Bars, ± SE.

7% cytotoxicity, further experiments on drug synergy, using the ICFA method, were carried out using 1000 U/mL of TNF or less. Figure 1 shows that TNF (1000 U/mL) when incubated simultaneously with different concentrations of Mitoxantrone (from 0.001 to 10 μM) for 1 hr potentiates, in a dose-dependent manner, the cytotoxic effect of the drug. The IC_{50} of Mitoxantrone alone was 0.0794 μM, while in the presence of TNF it was 0.0076 μM (potentiation ratio = 10.5).

Because 100 U/mL TNF is the most active dosage in terms of break potentiation we have also performed the ICFA assay combining different concentrations of Mitoxantrone (from 0.001 to 10 μM) with 100 U/mL TNF. Figure 1 also shows that TNF (100 U/mL) when incubated simultaneously with Mitoxantrone for 1 hr potentiates the cytotoxic effect of the drug. In this case, the IC_{50} of Mitoxantrone, in the presence of TNF (100 U/mL), was 0.0024 μM (potentiation ratio = 33.1). TNF at 100 U/mL was more active in inducing potentiation of Mitoxantrone cytotoxicity than at 1000 U/mL (about 3.2-fold).

Lack of effect of TNF on the cellular uptake and retention of Mitoxantrone

The uptake of [^{3}H]Mitoxantrone by A2774 cells was time and concentration dependent (Fig. 2A and B) and tended to level off slightly at concentrations above 3.2 μM. TNF (100 U/mL) does not modify the uptake of [^{3}H]Mitoxantrone (both time and concentration dependent) (Fig. 2A and B). TNF at 100 U/mL is the most effective dose in terms of cytotoxicity and break potentiation which is why we have used this dose. The effect of TNF (100 U/mL) on [^{3}H]Mitoxantrone retention in A2774 cells is shown in Fig. 2C. Retention of Mitoxantrone was similar in the presence and absence of TNF.

Potentiation by TNF of DNA SSBs induced by Mitoxantrone

The effect of TNF on DNA strand breakage induced by Mitoxantrone was evaluated. TNF alone (from 10 to 1000 U/mL) does not induce DNA SSBs when incubated with the cells for 1 hr (data not shown). Addition of TNF potentiates Mitoxantrone-induced DNA SSBs, showing a linear correlation with the concentration of Mitoxantrone (Fig. 3A and Table 1). The enhancement of Mitoxantrone-induced DNA SSBs by TNF is also dependent on TNF concentration. TNF, at all concentrations used, increased the number of DNA SSBs produced by Mitoxantrone, with 100 U/mL giving the maximum effect. TNF consistently increased the numbers of DNA SSBs generated by Mitoxantrone (Table 1). For example, in the absence of TNF, there are 82.2 rad equivalents of SSBs (1 hr exposure to 1 μM Mitoxantrone) in the A2774 cells compared to 406.2 rad equivalents in its presence (100 U/mL). In addition, while no SSBs are observed in A2774 cells treated with 0.01 or 0.1 μM Mitoxantrone (for 1 hr), in the presence of 100 U/mL TNF, 16.2 or 146.2 rad equivalents of SSBs are observed. The results of the elution experiments clearly demonstrate TNF-induced potentiation of "cleavable complex" formation generated following treatment with Mitoxantrone. The increase in DNA SSBs was not caused by increased drug accumulation after TNF treatment, as observed in [^{3}H]Mitoxantrone uptake studies (Fig. 2A). Mitoxantrone produced breaks that persisted for 2–3 hr following removal of cells from the drug-containing medium (Fig. 3B). In the presence of TNF (100 U/mL) these breaks were reversed with kinetics of disappearance similar to those seen for Mitoxantrone alone (Fig. 3B). The efflux rate of Mitoxantrone was also not affected by TNF (Fig. 2C).

When proteinase K and SDS were omitted from the assay procedure (filter binding assay) and PVC filters were used, no fragmentation was detectable in A2774 cells treated with 10 μM Mitoxantrone (Table 2). Similar results were also obtained when cells were incubated simultaneously with TNF (100 U/mL) and 10 μM Mitoxantrone for 60 min (Table 2). Therefore, all breaks detected under deproteinizing conditions are protein-linked breaks.

Table 1. Effect of TNF on protein-concealed DNA SSBs induced by Mitoxantrone

Mitoxantrone (μM)	DNA SSBs (rad equivalents)			
	−TNF	+TNF 10 U/mL	+TNF 100 U/mL	+TNF 1000 U/mL
0.01	0	0	16.2	46.7
0.10	0	0	146.2	73.2
1.00	82.2	161.2 (96.1)	406.2 (394.2)	226.2 (175.2)
10.0	167.2	251.2 (50.2)	606.2 (262.6)	331.2 (98.1)

A2774 cells were treated with Mitoxantrone for 1 hr at 37° with or without TNF and then assayed for DNA strand breaks by alkaline elution, under deproteinizing conditions [15].

Results are expressed in terms of the X-ray dose that would produce an equivalent elution in the SSB assay and the corresponding units are designated SSB rad equivalents. A2774 cells were treated with different dosages of γ-rays from a [137]Cs source to obtain a standard curve (data not reported).

In parentheses: percentage increases in DNA SSBs over the numbers produced by 1 hr incubation with Mitoxantrone alone.

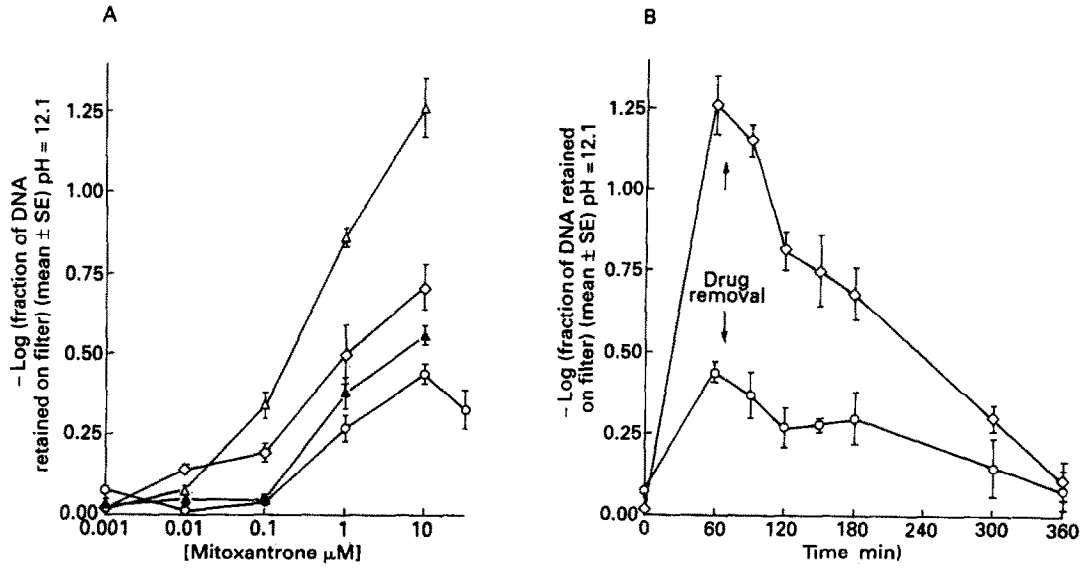


Fig. 3. (A) Effect of addition of TNF on DNA SSBs induced by Mitoxantrone. ^{3}H-Labeled A2774 cell monolayers were treated with medium only or Mitoxantrone (from 0.01 to 10 μM) in the presence [(▲) 10, (△) 100, (◇) 1000 U/mL)] or absence (○) of TNF for 1 hr at 37°. Cells were harvested and DNA SSBs were assayed by mean of the proteinase K-alkaline elution technique [15]. Data are representative of four or six separate experiments. Bars, ± SE. (B) Disappearance of DNA SSBs in A2774 cells treated with 10 μM Mitoxantrone in the presence (◇) or absence (○) of TNF 100 U/mL. Cells were incubated with drug at 37° for 1 hr. Drug was removed from the medium by washing and cells were incubated in fresh medium. DNA SSBs were quantified by proteinase K-alkaline elution. Data are representative of more than three separate experiments. Bars, ± SE.

Effect of TNF treatment on extractable topoisomerase II activity

Topoisomerase II activities can be extracted from isolated nuclei by incubating them at 4° in a buffer containing 0.35 M NaCl. Under conditions identical to those used in the DNA strand breakage assay, except for the omission of Mitoxantrone, TNF treatment induced a rapid and transient increase in extractable topoisomerase II activity (Table 3), with a maximum achieved 2.5 min after TNF treatment. After 10–30 min the activity was equal to, or lower than, that of untreated cells.

Table 2. Effect of TNF (100 U/mL) on Mitoxantrone-induced DNA fragmentation

Mitoxantrone (μM)	Fraction of fragmented DNA	
	−TNF	+TNF
0.0	0.17 ± 0.07 (N = 6)	0.18 ± 0.08 (N = 6)
10.0	0.19 ± 0.03 (N = 8)	0.17 ± 0.10 (N = 8)

Cells were treated as in Table 1. DNA fragmentation was determined by the filter binding assay under non-deproteinizing conditions [17].

Results are expressed as the fraction of fragmented DNA (relative to total DNA) and values are means ± SE (N, number of independent determinations).

Analysis of statistical significance according to non-parametric Mann–Whitney test [25] indicated $P > 0.520$ (not significant) for drug-treated cells versus controls (untreated cells).

Kinetics of TNF-induced potentiation of DNA SSBs and cell killing by Mitoxantrone

To ascertain whether the transient nature of the enhancement of extractable topoisomerase II activity induced by TNF is reflected in the potentiation of DNA SSBs induced by Mitoxantrone, we started the incubation with TNF (100 U/mL) 10 or 30 min before incubation with Mitoxantrone (1 hr). This resulted in abrogation of the TNF-mediated potentiation of DNA damage (Table 4). TNF-mediated potentiation of Mitoxantrone-induced DNA SSBs was achieved only when TNF was added simultaneously with the drug.

To determine further whether the TNF-induced transient increase of extractable topoisomerase II activity and potentiation of DNA SSBs were associated with the potentiation of cytotoxicity of Mitoxantrone, the effect of pretreatment with TNF on Mitoxantrone-mediated cytotoxicity was investigated by the ICFA test. A2774 cells were incubated for 10 or 30 min with TNF (100 or 1000 U/mL). After this initial treatment, excess TNF was partially removed by aspirating the supernatants, but without further washing of the cells. Different concentrations of Mitoxantrone (0.001–10 μM) were added to the cell cultures for an additional 1 hr. The results in Fig. 4 and Table 5 demonstrate that potentiation of Mitoxantrone cytotoxicity was found only when A2774 cells were treated simultaneously with TNF. No synergy was detected when cells were incubated with TNF for 30 min before treatment with Mitoxantrone (Fig. 4 and Table 5). Thus, pre-

Table 3. Effect of TNF treatment on extractable topoisomerase II catalytic activity

	Topoisomerase II catalytic activity† (U/mg total nuclear protein)				
		+100 U/mL TNF			
		Length of incubation (min)			
Experiment*	−TNF	2.5	5	10	30
1	75.0	116.0	100.0	60.0	51.0
2	91.0	141.0	116.0	75.0	67.6
3	78.0	121.0	101.0	81.0	60.0
4	93.0	162.0	133.0	68.0	58.0
Mean ± SE	84.3 ± 4.5	135.0 ± 10.5	112.5 ± 7.8	71.0 ± 4.5	59.2 ± 3.4

A2774 cells (0.7×10^7 cells/80 cm^2 flask) were incubated with TNF for the length of time indicated.

* Each experiment was performed with fresh nuclear extract (containing 200 ng total protein) prepared from exponentially growing cells in mid-log phase. Glycerol was added to a final concentration of 50% to each sample.

† Topoisomerase II catalytic activity was assayed quantitatively by the centrifugation method as described by Deffie *et al.* [19]. One unit of enzyme is defined as the amount that catalyses the release of 400 ng of DNA into the supernatant as free minicircles in 30 min at 30°.

Analysis of statistical significance according to non-parametric Mann–Whitney test [25] indicated the following: TNF at 2.5 and 5 min, more catalytic activity than without TNF ($P = 0.014$). TNF at 10 min, catalytic activity not significantly different than without TNF ($P > 0.557$). TNF at 30 min, catalytic activity higher without TNF than with TNF ($P = 0.014$).

exposure of A2774 cells to TNF alone (100 or 1000 U/mL) for time longer than 10 min, when the TNF-induced increase in topoisomerase II catalytic activity had reversed to less than control levels (Table 3), resulted in abrogation of the TNF-mediated potentiation of DNA SSBs and cytoxicity induced by Mitoxantrone.

DISCUSSION

Although significant progress has been made in the treatment of malignant diseases with chemotherapy, there are still diseases for which no effective chemotherapy exists, and there are many initially responsive tumors that become resistant to previously effective therapy. The combination of biological response modifiers and cytotoxic drugs offers a new approach to increasing the therapeutic index in the treatment of neoplastic diseases.

Biochemical modulation refers to the administration of a modulator to manipulate the metabolic pathway of a cytotoxic drug, resulting in the selective enhancement of antitumor activity. In contrast, biological modulation entails immunotherapy with cytokines and or immunocompetent cells in combination with cytotoxic drugs. Biochemical modulation of anticancer drug action does not generally

Table 4. Effect of preincubation with TNF on protein-concealed DNA SSBs induced by Mitoxantrone

TNF treatment before drug addition	DNA SSBs (rad equivalents)
None	167.2* (123.1)
TNF, 0 min	606.2* (262.6)
TNF, 10 min	176.8 (5.7)
TNF, 30 min	145.4 (−13.0)

A2774 cells were treated with TNF (100 U/mL) 0, 10 or 30 min before the addition to the incubation medium of Mitoxantrone (10 μM) and further incubation for 1 hr. TNF was not washed out from the cells before incubation with Mitoxantrone.

* As reported in Table 1.

In parentheses: percentage increases in DNA SSBs over the numbers produced by 1 hr incubation with Mitoxantrone alone.

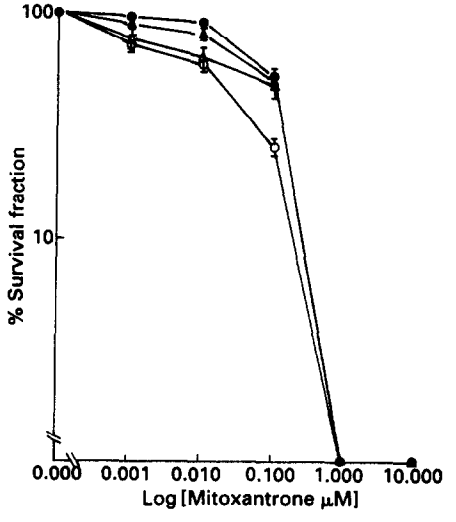


Fig. 4. Relative survival of human ovarian cancer cell line A2774, exposed to TNF 1000 U/mL 10 (△) or 30 (▲) min before treatment with various concentrations of Mitoxantrone for 1 hr at 37° in the clonogenic assay; or TNF 100 U/mL 10 (○) or 30 (●) min before Mitoxantrone. Compare with Fig. 1. Bars, ± SE.

Table 5. Effect of preincubation with TNF on Mitoxantrone cytotoxicity

TNF treatment before drug addition	IC_{50} (μM)	PI
None	0.0794	—
TNF 1000 U/mL, 0 min	0.0076	10.50
TNF 1000 U/mL, 10 min	0.0588	1.35
TNF 1000 U/mL, 30 min	0.1000	NP
TNF 100 U/mL, 0 min	0.0024	33.1
TNF 100 U/mL, 10 min	0.0156	5.1
TNF 100 U/mL, 30 min	0.1202	NP

A2774 cells were treated with TNF (1000 or 100 U/mL) 0, 10 or 30 min before the addition to the incubation medium of Mitoxantrone (10 μM) and further incubation for 1 hr. TNF was not washed out from the cells before incubation with Mitoxantrone.

IC_{50} = 50% inhibitory concentration.

PI, potentiation index: degree of potentiation by TNF expressed as a ratio of IC_{50} with and without TNF.

NP, not potentiated (perhaps protected).

imply an anticancer drug activity of the modulator, as is known from the various chemical modulators of the multidrug-resistance phenotype [20].

The reasons for combining cytokines and cytotoxic drugs to enhance antiproliferative activity are multiple and complex. There is convincing evidence of multiple levels of interaction between cytokines and cytotoxic drugs, including direct modulation of target enzymes of drug metabolism, modulation of receptors, modulation of cell cycle phases and modulation of gene expression, thereby altering the sensitivity to cytotoxic drugs. One of the most attractive approaches to biochemical modulation is the enhancement of topoisomerase II-targeted drug cytotoxicity by TNF.

This study demonstrates the potentiation of A2774 tumor cell killing by treatment with a combination of TNF and a topoisomerase II inhibitor, namely Mitoxantrone, and provides experimental evidence in support of possible molecular mechanisms that may be responsible for this synergistic activity. In addition, we suggest here that the effectiveness of the association is linked to specific timing of the administration of the two drugs.

It is important to note that TNF potentiates Mitoxantrone cytotoxicity as well as Mitoxantrone-induced DNA SSBs after 1 hr incubation, at TNF concentrations that are clinically achievable (<5000 U/mL) [21].

By using a filter binding assay, recently described by Bertrand *et al.* [17], which measures non-protein-linked DNA breaks, no DNA fragments were detected under non-deproteinizing conditions, for the combination Mitoxantrone + TNF. Since breaks induced by topoisomerase inhibition are protein-linked DNA breaks, not detectable under non-deproteinizing conditions [16], the observed potentiation of Mitoxantrone-induced DNA SSBs by TNF can only be interpreted as an effect on topoisomerase II. The mechanism of enhancement of Mitoxantrone-induced DNA SSBs is consistent with the transient increase in extractable topoisomerase II catalytic activities in TNF-treated A2774 cells. If, after TNF administration, we wait long enough for the disappearance of the effect on the extractability of topoisomerase II, the potentiating effect also disappears.

This augmentation of tumor cell killing by treatment with the combination of TNF and Mitoxantrone (1 hr at 37°) was not caused by increased drug accumulation after TNF treatment. Also the efflux of Mitoxantrone was not affected by TNF.

Branellec *et al.* [22], working with a WEHI164.13 murine fibrosarcoma cell line, showed that the potentiating effect of TNF on VP16 cytotoxicity (24 hr of drug treatment) was not accompanied by an increase in VP16-induced SSBs. Because we have found that two other lines originated from human ovarian tumors [23] and two primary cultures from human ovarian tumors [24] were regularly sensitive to the potentiating effect of TNF (in association with different topoisomerase II inhibitors), both in terms of cell killing and induction of DNA breaks, we suspect that the mechanism of interaction between TNF and topoisomerase inhibitors can sometimes be different in cells originated from different tissues.

Preincubation of A2774 cells with TNF for 10 or 30 min before the addition of Mitoxantrone resulted in no increase in DNA SSBs and no increase in cytotoxicity. Thus, kinetically, a cause–effect relationship may exist between TNF-induced increased activation of extractable topoisomerase II and enhanced DNA damage and cell killing by Mitoxantrone. Mitoxantrone should be present simultaneously with TNF in order to form a larger amount of topoisomerase II–Mitoxantrone complex, and to generate a larger amount of protein-concealed DNA SSBs. Any delay in the addition of Mitoxantrone would abolish the potentiating effect of TNF.

Our study may lead to a completely new therapeutic dimension in respect to many open questions such as: (1) which tumors are susceptible to the drug potentiating effect of TNF? (2) What is the proper timing of TNF in the combination? (3) What is the proper concentration/dose of TNF in the combination? In combining TNF with topoisomerase II-targeted drugs the timing of the association appears crucial for synergistic antiproliferative interactions. Pretreatment with TNF does not make tumor cells more vulnerable to subsequent killing by topoisomerase II inhibitors, but coincident treatment with TNF increases very significantly the drug-mediated cell killing. Optimization of the dose of TNF may also be necessary for a maximum potentiation of the cytotoxicity of topoisomerase II inhibitors; 100 U/mL (both in terms of cytotoxicity and DNA breaks) appear already sufficient. Higher concentrations are not needed. This behaviour could have an equivalent even in *in vivo* situations.

Finally, an alternative therapeutic approach to human ovarian cancer, in patients relapsed after standard chemotherapy might be the use of TNF in combination with Mitoxantrone or VP16 [6]. These data may be relevant to the planning of future animal and human trials using TNF and topoisomerase II-targeted drugs. In conclusion, we believe that the

combination TNF + Mitoxantrone has potential for the intraperitoneal therapy of relapsed human ovarian cancers, and we are currently planning phase I/II clinical trials to investigate this possibility.

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